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IDENTIFICATION OF GENETIC VARIANTS AND THEIR IMPLICATIONS IN AUTOIMMUNITY

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Identification of genetic variants and their implications in autoimmunity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Pulling the puzzles apart
Questions of science
Science and progress

Nobody said it was easy
No one ever said it would be so hard

The Scientist by Coldplay

ABSTRACT

Autoimmune disorders start to develop when the body's immune system recognizes organs and tissues as foreign and initiates uncontrolled immune reactions against them. Most of these disorders are regarded as complex with both environmental and genetic factors contributing to disease development. Current treatment of autoimmune disorders such as Rheumatoid arthritis (RA) is associated with lack of efficacy, development of resistance and serious side-effects and accentuates the need for development of new therapeutics. Improved understanding of the underlying genetic pathways that convey pathogenicity in arthritis is key to discover more efficient and safe therapies. The heterogenetic nature of autoimmune diseases and the interaction with environmental factors delays the discovery of susceptibility genes in humans, which suggests the use of animal models where both genetic background and environment can be controlled. In this thesis we have used rat models to identify genes that regulate the induction of autoimmune arthritis. In study one, we identify the gene encoding Endophilin A2 as a major determinant in regulating the induction of autoimmunity and show that the Endophilin A2 mediated protection is regulated via T cell responsiveness. In study two, we investigate the role of the *Vav1* gene, previously associated to multiple sclerosis, for its role in arthritis in rats and humans and show that natural variants in the *Vav1* gene regulate T cell dependent arthritis. In study three, we determine by functional studies that the increase in reactive oxygen species conveyed by the *Ncf1* gene, is responsible for reduced arthritis severity seen in *Ncf1* congenic rats. In study IV, we use high resolution mapping in a rat heterogeneous stock to identify genes regulating expression of cell surface molecules and frequency of different leukocytes in blood. By combining animal studies and human data we have in this thesis identified new genes involved in the pathogenesis of arthritis, which further illustrates the heterogenic nature of RA and the shared peripheral tolerance pathways regulating different autoimmune disorders. Furthermore, the results in this thesis have demonstrated the value of using animal studies to identify genes and pathways relevant to human disorders.

LIST OF SCIENTIFIC PAPERS

- I. **Spontaneous mutation reveals Endophilin A2 as a major regulator of autoreactive T cells and a potential new target in autoimmune disease.**

Ulrika Norin, Carola Rintisch, Florian Forster, Liesu Meng, Diana Ekman, Jonatan Tuncel, Katrin Klocke, Johan Bäcklund, Min Yang, Klementy Shchetynsky, Hanna Axelsson, Martin Haraldsson, Thomas Lundbäck, Maria Bergquist, Leonid Padykov, Inger Gjertsson, Pietro de Camilli, Norbert Hubner, Liselotte Bäckdahl, Rikard Holmdahl
Manuscript

- II. **VAV1 regulates experimental autoimmune arthritis and is associated with anti-CCP negative rheumatoid arthritis**

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- III. **Positioning of a Polymorphic Quantitative Trait Nucleotide in the Ncf1 Gene Controlling Oxidative Burst Response and Arthritis Severity in Rats**

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- IV. **Combined sequence-based and genetic mapping analysis of complex traits in outbred rats**

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LIST OF ABBREVIATIONS

RA	Rheumatoid Arthritis
MS	Multiple Sclerosis
MHC	Major histocompatibility complex
TCR	T cell receptor
PTM	Post-translational modifications
ROS	Reactive-oxygen species
mTEC	medullary thymic epithelial cells
DC	dendritic cell
MQ	macrophage
APC	antigen-presenting cell
AIRE	autoimmune regulator
TRA	Tissue- restricted antigen
TGFb	Transforming growth factor beta
IL-10	Interleukin 10
GI	gastrointestinal
RF	Rheumatoid factor
ACPA	anti-citrullinated protein antibody
DMARD	Disease-modifying antirheumatic drug
PIA	pristane-induced arthritis
CIA	collagen-induced arthritis
GPIA	glucose-6-phosphate isomerase- induced arthritis
QTL	quantitative trait loci
SNP	single nucleotide polymorphism
CNV	copy number variation
GWA	genome-wide association
HS	heterogeneous stock
Mb	Mega base pairs
Kb	Kilo base pairs

1 INTRODUCTION

To keep us safe from infections and cancer our immune system has evolved to recognize and neutralize antigens foreign to us, such as pathogens and altered self-antigens present in cancer cells. First line of defense includes the skin barrier and the mucous membranes that prevent pathogens from entering our bodies and the innate immune system. The innate immune system is a rapid acting defense system that contains cells like the neutrophils, macrophages and dendritic cells and the complement system. These have evolutionary conserved molecules and receptors that recognize structures foreign to us, like infectious agents from bacteria or viruses, to ensure immediate neutralization and clearance of the pathogens. The second line of defense is the adaptive immune system, which includes the lymphocytes, B cells and T cells. Unlike the innate immune system, the lymphocytes function like an immunological memory and upon recognition of a pathogen they would become activated and a subset of the cells will become long-lived memory cells that can respond quickly if the infection was to happen again. Because they are long-lived the response needs to be tightly regulated since a wrongfully directed response towards for example a self-antigen could have detrimental consequences as seen in individuals with autoimmunity. To ensure that the response to a certain antigen is correct, the innate and the adaptive immune system communicate via surface molecules and cytokines in order to distinguish between potential harmful and safe events.

In this thesis I have studied some of the genes involved in in this intricate interplay of the immune system. Using animal models for common autoimmune disorders such as Rheumatoid arthritis and multiple sclerosis we have determined the functional implication the genes have in regulating the immune response and how disturbances in this regulation can lead to autoimmunity. In paper I and II we investigate the functional impact that T cells have on autoimmunity and in paper III how reactive oxygen species can alter the autoimmune response. Understanding the mechanisms of these pathways is key to develop better and safer therapies for autoimmune disorders.

2 AUTOIMMUNE DISORDERS

Autoimmunity is a condition where the body mounts an attack against the body's own tissues and organs and is characterized by the presence of autoantibodies and T cells reactive to self-antigens. Autoimmunity affects a large proportion of the Western population with an estimated prevalence of 7.6–9.4%¹. Autoimmune disorders can be organ restricted as in thyroiditis, type I diabetes and multiple sclerosis or systemic, affecting several organs, such as systemic lupus erythematosus and rheumatoid arthritis. There is a strong genetic component to autoimmune disorders seen as familial aggregation of autoimmunity in affected individuals. However, predisposition is not only determined by the genetic make-up of an individual but include environmental factors as well. Thus, they are considered complex disorders where many factors determine if an individual will develop autoimmunity. The induction of complex disorders can be illustrated using the threshold liability model². Here a set of genetic factors and environmental factors will contribute to the disease pathogenesis and the subsequent contribution of all factors will determine if an individual will cross the threshold and manifest with a clinical disease³, Fig. 1.

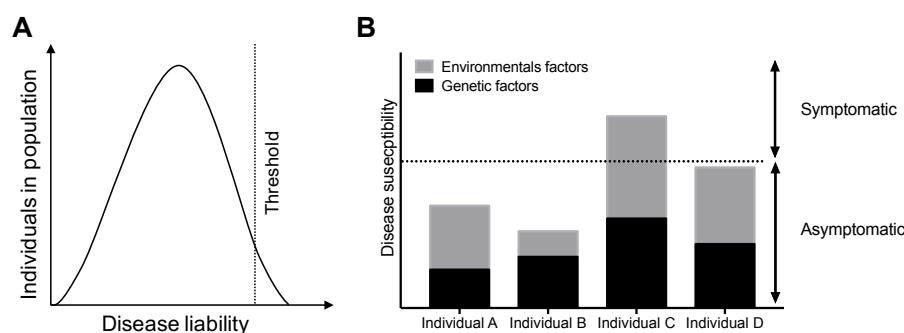


Figure 1. Threshold model for autoimmune disorders. A) Normally distributed genetic liability for an autoimmune disorder in a population. Adapted from Haegert³ B) Expanded model of susceptibility including environmental factors which influence disease susceptibility in individuals..

Genetic and environmental factors can increase the relative risk of developing disease but they can also have a protective effect⁴. The phenomenon that autoimmune disorders are complex is further illustrated by the fact that monozygotic twins, although sharing an identical genome, do not necessarily have the same risk of developing autoimmunity. Rather than being 100%, the concordance rate for autoimmune disorders in monozygotic twins range from 12% in RA to 75% in Ankylosing spondylitis⁵. Still, the relative risk of developing autoimmunity is higher in relatives to affected individuals than the general population. Thus, there is a strong genetic link in predisposing individuals to autoimmunity however additional external triggers/factors are also needed.

Although the targeted organ and subsequent functional outcome is different between different autoimmune disorders they share common predisposing genetic pathways and autoimmune disorders aggregate within families⁶. The most dominant genetic contributor is the major histocompatibility complex (MHC). Genes encoding MHC molecules are by far the most associated genes to autoimmune disorders. The purpose of the MHC molecules is to present peptides to T cells, either by presenting extracellular antigens as is the case of the MHC class II molecules or intracellular antigens as in the MHC class I molecules. Different MHC genes associate to different autoimmune disorders and could indicate that the presentation of a specific antigen on a certain MHC molecule or the expression of a certain MHC molecule influence the targeted response to a specific organ⁷. Other shared loci regulating predisposition to autoimmunity has also been identified. Notably many of them affecting T cell mediated immune functions, implicating T cells as major determinant of autoimmune predisposition⁸. Direct targeting of one of these genes, the *CTLA4* gene, encoding the cytotoxic T-lymphocyte-associated protein 4 is currently being used in the treatment of autoimmune disorders⁹.

As the environment around us shapes the immune system and its response, the fact that environmental factors can influence the predisposition to autoimmune disorders is logical¹⁰. This notion is maybe best exemplified in celiac disease where the addition of gluten in the diet of genetically susceptible individuals leads to an autoreactive response to transglutaminase-2 and induction of T cell driven destruction of the small intestines. Exclusion of gluten leads to an immediate cessation of the autoreactive response and the lesions of the intestines heal¹¹.

Lately the importance of immune system homeostasis influenced by the microbiota¹², and particularly the microflora in the gastrointestinal (GI) tract, has been getting much attention. Increasing evidence shows that the presence of different commensal bacteria can shift the balance between regulatory and disease driving T cells. The direct influence of the gut microflora in the development of autoimmunity has been shown by use of the segmented filamentous bacteria (SFB) in the K/BxN mouse model. Here they showed that introduction of a single pathogen into germ free mice increased the number of disease driving Th17 cells and led to the induction of autoreactive responses¹³. Another mechanism by which infections can cause autoimmunity can be explained by molecular mimicry¹⁴. Here an antigen from a pathogen is structurally similar to an antigen present in the endogenous body and thus upon infection the immune reaction will be misguided towards a self-antigen and lead to destruction of the target tissue.

Molecular mimicry has been suggested to trigger autoimmunity in a variety of disorders for example cytomegalovirus leading to cross-reactivity in Type-I diabetes¹⁵ and cross-reactive immune response to streptococcal M protein and cardiac myosin in rheumatic heart disease¹⁶.

In summary, the overall induction of autoimmunity is determined by genetic and environmental factors and dependent of immune-reactivity, antigen recognition and tissue modulation of the immune response. The autoimmune response can be seen years before any signs of clinical disease¹⁷ and the identification of the dysregulated immune response early on is key to improve the outcome for the patients.

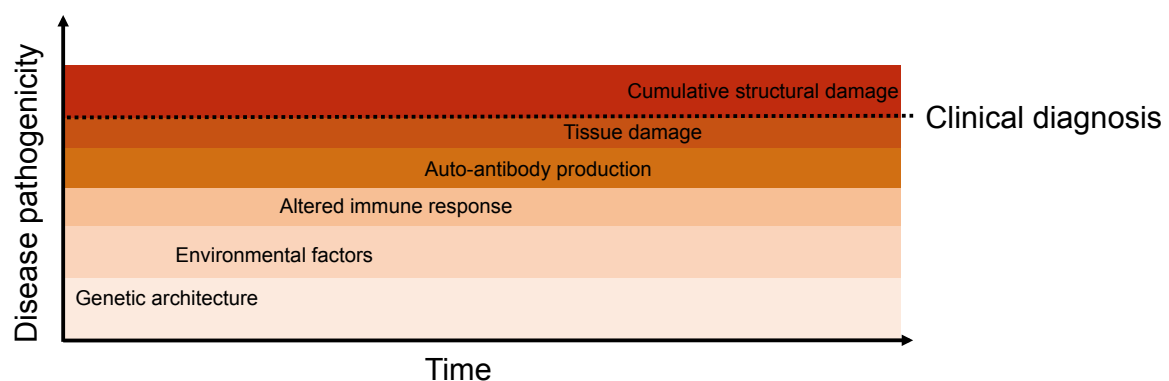


Figure 2 Development of autoimmunity. Environmental exposure in genetically predisposed individual lead to an altered immune reactivity towards self. Subsequent tissue damage leads clinical diagnosis. Adapted from Cho and Feldman¹⁸

2.1 Tolerance mechanisms to prevent the induction of autoimmunity

In order to prevent an aberrant autoreactive immune response, the immune system has developed a number of means to induce tolerance towards self- antigens. In this thesis we show that T-cells (papers I and II) have a specific role in regulating the development of autoimmunity. To eliminate self-reacting T cells two distinct processes have evolved, central tolerance that occurs in the thymus during T cell development and peripheral tolerance which occur out in the tissues. Disruption in either of the pathways can lead to autoimmunity.

2.1.1 Central tolerance

Most of the self-reacting and potentially dangerous T cells are deleted during development in the thymus. This process needs to be carefully balanced to include deletion of high affinity self-reacting T cells while ensuring there is enough variation (T cell clones) to allow for detection of foreign pathogens. It is estimated that out of all the T cell progenitors in the thymus only 5% eventually mature into T cells and enter the periphery¹⁹.

The maturation stages of the developing T cell are divided into two stages termed positive and negative selection. During positive selection, immature thymocytes are selected for their ability to produce a T cell receptor (TCR) that can recognize peptide bound to MHC molecules. A functional TCR will lead to induction of further survival and maturation signals while an inability to express a functional TCR will lead to death by neglect²⁰. Mature thymocytes are then negatively selected if they recognize self-peptides:MHC with high affinity²¹. These processes ensure that the T cells entering into the periphery are able to recognize self-MHC (and thus possible pathogenic peptides presented on them) but not MHC molecules bearing self-antigens.

The presentation of self-antigens during negative selection is dependent on medullary thymic epithelial cells (mTECs) expressing tissue-restricted self-antigens (TRAs) and antigen-presenting cells (APCs) loaded with antigens from the periphery migrating into the thymus^{22,23}. The importance of expression of self-antigens during negative selection in preventing autoimmunity is illustrated in humans and mice with mutations in the autoimmune regulator (*AIRE*) gene. *AIRE* is a transcription factor and regulates the expression of TRAs in mTECs and loss of function of *AIRE* leads to multi-organ autoimmunity²⁴.

The presence of auto-reactive T cells in healthy individuals implies that negative selection is incomplete²⁵ and could explain why some individuals develop autoimmune disorders. The incomplete negative selection could be due the lack of specific post-translational modification of certain proteins in the thymus²⁶. For example, an increased T cell response toward the citrullinated version of collagen type II compared to its native form can be seen in RA patients²⁷. Additionally, restrictions in the presentation of only one segment of a protein in thymus will lead to an incomplete tolerance towards the entire protein and can lead to susceptibility to autoimmunity²⁸.

2.1.2 Peripheral tolerance

Due to the limited expression of self-peptides in thymus the escape of self-reacting T cells is inevitable. Therefore mechanisms to keep self-reacting T cells under control in the periphery are crucial. This process is regulated within the T cell itself through ignorance and anergy and extrinsically by immune suppression. First the T cell needs to recognize the antigen and thus if the abundance of antigen is too low²⁹ or if the T cell is physically separated from a particular antigen, like the antigens in an immune privileged site like the eye³⁰ it will not be activated. Second, if an antigen is presented to a T cell, the T cell will also require co-stimulatory molecules³¹ to be activated or it will go into an unresponsive state called anergy. Additionally, ligation of molecules like CTLA-4³² or PD-1³³ on the T cells can actively induce anergy by inhibiting cell division and cytokine secretion. The third important pathway of limiting an autoimmune response is immune suppression. Specialized T cells, called regulatory T cells (Tregs), have a critical part in immune suppression. These are characterized by the expression of the *FOXP3* transcription factor and high levels of the anti-inflammatory cytokines IL-10 and TGF β and are important in inhibiting autoimmunity³⁴. The important role of Tregs has been shown to be evident in humans with the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) disorder. Here, mutations that cause a loss of function in the *FOXP3* transcription factor causes aggressive autoimmunity as a result of defects in the function of the regulatory T cells³⁵. Other cells such as tolerogenic APCs are also important mediators of immunosuppression and can limit T cell proliferation and activity³⁶. High levels of anti-inflammatory cytokines in tissues can also suppress T cell responses. For example, presence of high levels of IL-10 in the GI tract keeps the immune system in check even with a high amount of commensal bacteria and blockade of IL-10 leads to colitis in mice³⁷.

In this thesis we found three different ways by which peripheral tolerance mechanisms limit autoimmunity. In paper I, a reduced antigen-specific response of the peripheral T cells is displayed as a consequence of decreased signaling from the TCR complex, leading to protection against autoimmunity. In paper II a polymorphism in the T cell signaling molecule VAV1 leads decrease in T cell effector functions which subsequently leads to a reduction in severity of arthritis. In paper III an increased production of reactive-oxygen species (ROS) by APCs change the arthritogenic T cell response³⁸ and lead protection of arthritis.

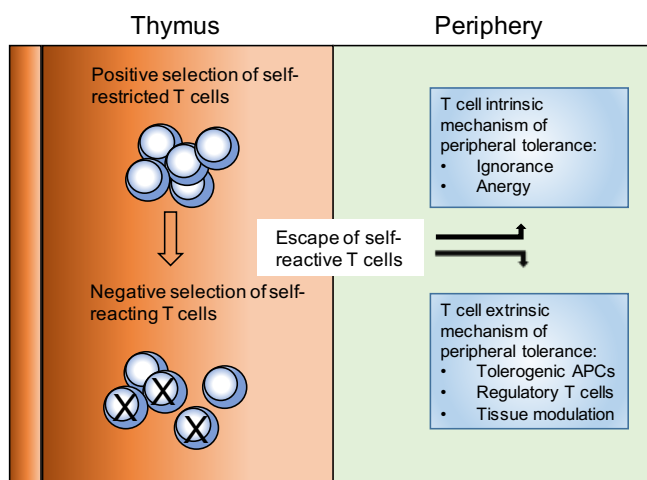


Figure 3. Pathways of central and peripheral T cell tolerance. Hematopoietic progenitors migrate from the bone marrow to the thymus where they mature to T cells and undergo positive and negative selection based on their interactions with peptide-MHC molecules. Self-reactive T cells that fail to undergo deletion are controlled in the periphery by intrinsic and extrinsic peripheral mechanisms. Adapted from Walker and Abbas³⁹.

3 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease, characterized by chronic destruction of synovial joints subsequently leading to loss of function of the joints. The term Rheumatoid arthritis was coined by Sir Alfred Garrod in 1850s and is not a disease of the modern society but has affected humans for hundreds to thousands of years^{40,41}. RA affects approximately 0.5-1% of the human population today and affects females three times more often than males and disease onset is at around 30-60 years of age. As in other autoimmune disorders both genetic and environmental factors predispose individuals to RA but the precise cause of RA is still not known. RA primarily affects the synovial joints but systemic immune responses lead to other extra-articular manifestations such as rheumatoid nodules, pulmonary and cardiovascular diseases and is regarded a systemic disorder⁴². Individuals suffering from RA has greatly reduced quality of life and they have a shorter life expectancy compared to the general population⁴³. As of yet there is no cure and thus current treatment is focused on treating the symptoms.

3.1 *Clinical features and diagnosis*

RA is diagnosed according to the ACR/EULAR classification criteria (Table 1)⁴⁴ and is characterized by leukocyte infiltration into the synovial joint with subsequent inflammatory response resulting in cartilage and bone destruction⁴⁵. The joint of the hands, the wrists, and small joints of the feet are most commonly affected with subsequent involvement of the joints in the hips and shoulders as the disease progress. RA patients are routinely divided into serological positive and negative patients based on the presence of Rheumatoid factors (RFs) and anti-citrullinated protein antibodies (ACPAs). Due to the highly predictive value of the presence of ACPAs in diagnosis RA with a sensitivity of around 60%⁴⁶, ACPA positive RA patients are often diagnosed and treated earlier compared to ACPA negative patients. The pathogenesis of the two subtypes of RA patients appear to be different in terms of predisposing genetic and environmental factors, discussed in more detail below, and ACPA positive patients are often described to have a more erosive disease course⁴⁷.

Table 1. ACR/EULAR 2010 criteria for diagnosing RA	
Score points are shown in parentheses. A score of six or higher is required for RA diagnosis	
1. Joint involvement (0–5)	<ul style="list-style-type: none"> • One medium-to-large joint (0) • Two to ten medium-to-large joints (1) • One to three small joints (large joints not counted) (2) • Four to ten small joints (large joints not counted) (3) • More than ten joints (at least one small joint) (5)
2. Serology (0–3)	<ul style="list-style-type: none"> • Negative RF and negative ACPA (0) • Low positive RF or low positive ACPA (2) • High positive RF or high positive ACPA (3)
3. Acute-phase reactants (0–1)	<ul style="list-style-type: none"> • Normal CRP and normal ESR (0) • Abnormal CRP or abnormal ESR (1)
4. Duration of symptoms (0–1)	<ul style="list-style-type: none"> • Less than 6 weeks (0) • 6 weeks or more (1)

Co-morbidities are common in RA and include infections, cardiovascular disease, malignancies (most often lymphomas) and depression⁴⁸. Some are a consequence of the ongoing chronic inflammation but can also be present before or in conjugation with clinical onset. The increase in premature death in RA patients has been linked to co-morbidities and especially infections and cardiovascular disease⁴⁹.

3.2 Treatment

There is currently no cure for RA and available therapy for reversing the destruction of the cartilage and bone is missing. Thus, the standard treatment is focused on limiting the inflammatory response (Table 2)⁵⁰. Early and aggressive treatment strategy for a good outcome is necessary⁵¹ and for better management of the disease a scheme for treatment has been formed⁵². Initial treatment is initiated with disease-modifying antirheumatic drugs (DMARDs) such as hydroxychloroquine, leflunomide and methotrexate and depending on disease activity is supplemented with biological agents such as TNF blockers or anti-CD20. The first line of treatment most commonly used is the folate antagonist, methotrexate. It is thought to inhibit proliferation of cells by inhibiting the synthesis of pyrimidine and purine, the building blocks of DNA and RNA. Methotrexate has also been shown to inhibit cytokine production and decrease expression of adhesion molecules⁵³. The most commonly used biological agents are the TNF α blockers. TNF α can be found in high levels in the rheumatic joints and regulates the expression of other cytokines such as IL-1 and IL-6⁵⁴. TNF α seems particularly important in arthritis pathogenesis and transgenic mice expressing continuous levels of humanized TNF α develops spontaneous and chronic arthritis⁵⁵. TNF α blockade is effective in a majority of RA patients but has shown to increase the risk of infections⁵⁶.

Table 2. Frequently used therapeutics in RA

Conventional DMARDs	Mechanism of action
Methotrexate	Folate antagonist, inhibits cell proliferation, cytokine production
Leflunomide	Dihydroorotate dehydrogenase inhibitor, inhibits pyrimidine synthesis, NFkB activation, TNFa and matrix metalloproteinases production
Hydroxychloroquine	Inhibits B- and T cells activity and cytokine release
Sulfasalazine	Folate antagonist, inhibits acachidonic acid cascade
Tofacitinib	JAK1/2/3 inhibitor, inhibits cytokine production
Biological DMARDs	
Adalimumab- Human monoclonal antibody	TNF inhibitor
Certolizumab pegol- F(ab') fragment of a humanised monoclonal antibody	TNF inhibitor
Etanercept- IgG–Fc-receptor construct (fusion protein)	TNF inhibitor
Golimumab- Human monoclonal antibody	TNF inhibitor
Infliximab- Chimeric monoclonal antibody	TNF inhibitor
Rituximab- Chimeric monoclonal antibody against CD20	B- cell depletion
Abatacept- IgG–Fc-receptor construct (fusion protein)-CTLA4	Anti-T-cell co-stimulation
Tocilizumab	IL-6 inhibitor
Corticosteroids	
prednisolone	Inhibits acachidonic acid cascade

Table adapted from Smolen et al⁵⁷, Allan Gibofsky⁵⁰ and Sardar and Andersson⁵⁸

3.3 Predisposing genetic factors in RA

Using familial studies the heritability for RA has been estimated to about ~65%⁵⁹ and suggest a strong genetic component to RA. The shared genetic predisposition between RA patients was first described in 1976 by Peter Statsny⁶⁰. Through mixed lymphocyte reactions Stasny showed that cells from RA patients produced a low response toward RA stimulatory cells whereas a normal allogenic response was observed towards non-RA controls. Stasny believed that this reflected an association to the MHC molecule and showed that the frequency of the MHC gene HLA-DR4 was increased in RA patients. The association was indeed later confirmed to be the HLA-DR4^{61,62}. Many HLA-DR alleles have been associated to RA and led to the *shared epitope* hypothesis⁶³ suggesting a shared molecular structure in T cell recognition of the MHC molecule. The associated HLA-DRB1, HLA-B and HLA-DP in RA have subsequently been shown to share a particular five amino acid sequence, in the peptide binding groove of the of the associated molecules⁶⁴ and explain most of the genetic association in ACPA positive patients. It would take many years after the identification of the HLA-DR genes before another gene could be convincingly linked and associated to RA,

namely the *PTPN22* gene⁶⁵. Since then many loci have been identified using whole genome associations studies (GWAS)⁶⁶ with most genes directly linked to immune regulatory functions.

While the concordance rates between twins in both ACPA positive and negative RA patients have been reported to be equal⁵⁹, many other studies report a lower heritability in the ACPA negative individuals⁶⁷. Additionally, different genetic⁶⁸ and environmental⁶⁹ associations suggests that the predisposition in the two different subtypes might be different. Our studies in paper II seems to agree with this notion. Here an association with *VAV1* is found with the ACPA negative subgroup but not in the ACPA positive group. Additionally, we could show that the gene regulates the arthritis severity in a B-cell independent arthritis model but not a B-cell dependent model.

3.4 Predisposing environmental factors in RA

Smoking is by far the most well-known environmental factor in RA. The presence of ACPAs, shared epitope alleles and smoking increase the relative risk by up to 40% in developing RA⁷⁰. No such association has been found in ACPA negative RA patients suggesting that the two subtypes may have different environmental triggers. Some additional factors have been associated to increased risk of RA include periodontitis⁷¹ and microbiota in the gut⁷² while other such as alcohol intake and high birth-weight might decrease the risk of developing RA⁷³. Interestingly, occupational exposure to mineral oils, and in particular hydraulic oil, have been shown to be associated to RA⁷⁴. A known constituent of hydraulic oil is pristane⁷⁵ the same oil used to induce arthritis in rats.

3.5 Experimental models of RA

RA is a heterogeneous disease and there are many animal models for mimicking different aspects of the disease development and they can be spontaneous or induced^{58,76}. Animal models are great tools for investigating the cause and consequence of different genetic and environmental factors in arthritis pathogenesis. Pathways that regulate arthritis development are shared among species and arthritis regulating genetic loci found in rodents overlap with regions found in human studies, like the MHC region, proving their usefulness in identifying new targets for therapeutics. For example, the development of the new IL23/12 bi-specific antibody in treating autoimmune disorders is based on a finding in mice⁷⁷. The need for animal models in testing new therapeutics is also of importance⁷⁸. In this thesis we have used three different animal models for RA; collagen-induced arthritis (CIA), pristane-induced arthritis (PIA) and glucose-6-phosphate isomerase- induced arthritis (GPIA).

3.5.1 Collagen-induced arthritis

CIA is by far the most commonly used model for RA and can be induced in mice⁷⁹, rats⁸⁰ and non-human primates⁸¹. Immunization with the cartilage restricted protein collagen type II emulsified in either incomplete or complete Freund's adjuvant lead to immune response directed towards the joints and subsequent arthritis. Induction of CIA elicits leukocyte infiltration to the synovium, which leads to synovitis and pannus formation and cartilage and bone destruction. Both B- and T cells are required for disease induction, reviewed in⁸² and serum transfer can induce arthritis in both mice and rats indicating a strong contribution of antibodies in CIA pathogenesis^{83 84}. Like in RA, CIA is also highly dependent of the MHC⁸⁵ but also non-MHC genes⁸⁶. Immune reactivity to collagen type II is also found in RA patients both with antibody responses and auto-reactive T cells^{87,88}.

3.5.2 Pristane-induced arthritis

A single injection at the base of the tail of the mineral oil pristane ((2,6,10,14 tetramethylpentadecane)) induce a chronic relapsing arthritis in rats. Like in RA, immunization cause a symmetrical joint inflammation within twelve days with infiltrating leukocytes into the synovium subsequent synovial inflammation and production of rheumatoid factors⁸⁹. Why immunization with pristane induces arthritis is not known but is thought to be dependent on the polyclonal activation of self-reactive CD4+ T cells. Contrary to CIA, PIA cannot be transferred by serum and is thought to be less dependent on B cells⁹⁰. However, auto-antibody responses towards hnRNP-A2 and collagen type IX have been identified^{91,92}. Auto-reactive responses to these antigens have also been found in RA patients and could indicate common mechanistic pathways in the two disease^{93,94}. Very low amounts of pristane cause arthritis and the incidence is one hundred percent in the DA rats⁹⁵. Using blocking antibodies PIA has been shown to be dependent of T cells and can be adoptively transferred by MHC class II restricted CD4+ T cells⁹⁶. PIA can also be induced in mice however it appears to have a different induction pathway. To induce arthritis, the oil must be injected intraperitoneal and the arthritis starts much later at around 50 days and includes lupus like symptoms^{97,98}.

3.5.3 *Glucose-6-phosphate isomerase- induced arthritis*

Induction of arthritis by GPI was discovered as a consequence of a TCR transgenic mouse introgressed into the NOD mouse⁹⁹. The transgenic mouse produces large amounts of antibodies towards GPI and the disease can be transferred by serum, this model is called K/BxN model¹⁰⁰. Why an immune reaction towards GPI, which is a ubiquitous protein, leads to arthritis is thought to be because GPI is deposited in large amounts in the joints and antibodies towards GPI elicit complement activation and subsequent immune reaction locally in the joint¹⁰¹. In our model we immunize mice with a peptide from the orthologous human protein emulsified in complete Freund's adjuvant. This leads to monophasic arthritis with high incidence. It is a fast model with disease onset around day ten and has been shown to be dependent on both B and T cells as shown in B- and T-cell knock out mice¹⁰². GPI appear to be relevant to RA pathogenesis and autoantibodies towards the protein and be found in RA patients¹⁰³.

4 GENETIC DISSECTION OF COMPLEX DISORDERS

The theory that certain traits are inherited in distinct patterns were initially illustrated by Gregor Mendel in 1850s where he could show that certain phenotypic traits of peas were inherited from parental plants to daughter plants in either recessive or dominant form. While the traits Mendel investigated were due to single genes, this is not the case for complex disorders like RA. Here many genes of small effect sizes contribute to the phenotype. Identification of disease-regulating genes are further complicated by gene-gene and gene-environment interactions. Regions that are associated with a particular phenotype, commonly entitled quantitative trait loci (QTL), can be identified by linkage and association studies. In linkage studies, co-segregation of a particular trait with genomic loci in families of affected and unaffected individuals are identified. In association studies, the frequency of an allele or genotype in larger populations of unrelated affected and non-affected individuals is compared. If the genotype is over-representative in affected individuals the investigated genetic marker is believed to be associated to the trait. There are different types of genetic variants and polymorphisms that can affect genes and regulate a disease phenotype. In this thesis we describe an insertion of a transposon, which reduces the expression of a gene and coding single nucleotide polymorphisms (SNPs) that alter the function of the encoded proteins. Other genetic variants such as copy number variations (CNVs), as seen in regulation of the *NCF1* gene in humans¹⁰⁴, are also important in regulating gene function. Using different linkage and association studies, we have identified four different genes involved in immune regulatory phenotypes.

4.1 *Identifying disease causing genes in experimental crosses*

Experimental crosses of inbred strains of susceptible and resistant strains can be used to identify disease-regulating genes. A successful outcome of using animals for identification of disease regulating genes that are important in the human condition depends on both the phenotypic coherence between human and animal and the phenotype regulating genetic variability in the animals used. In paper I-III we used F2 crosses to identify genomic regions that regulate susceptibility to arthritis. Here offspring of one susceptible and one resistant strain called filial 1 generation (F1) are bred together to produce a second filial generation (F2). Each offspring in the F2 generation will have a unique mix of susceptible and resistant genomic loci spread across the genome, due to recombination events during meiosis. Using the information of markers that are polymorphic between the resistant and susceptible strain and by detecting the phenotype in each of the offspring one can perform a linkage analysis. To identify loci that are linked to the trait the logarithm of odds (LOD) score method is used¹⁰⁵. It is calculated by comparing the probability that a given marker is

inherited together with the trait, and thus are linked, with the probability of observing the same linkage by chance. Due to the limited number of recombination events in a F2 cross, the disease regulating genomic loci cover large genomic regions containing many genes and thus further isolation of the genetic loci in congenic strains through backcrossing is usually needed in order to identify the disease-regulating gene¹⁰⁶.

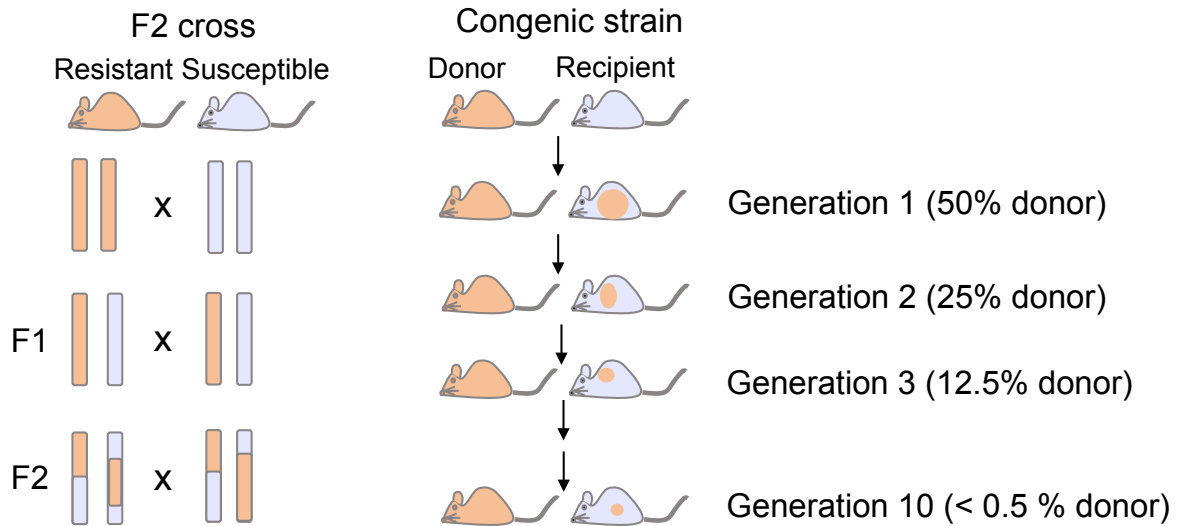


Figure 4. F2 crosses and congenic strains used to identify disease-regulating genes.

Nevertheless, to assign a phenotype to a single gene, known as positional cloning, using only congenic strains is difficult and time consuming and the use of functional studies and genetically modified animals might be needed. Using this combined approach, we could positionally clone the *SH3g1* gene in paper I and the ROS regulating nucleotide in the *Ncf1* gene in paper III.

As standard F2 genetic crosses only include the genetic variation of two parental strains and have low mapping resolution, we utilized a heterogeneous stock (HS) in paper IV. The NIH-rat HS was established using eight founding rat strains (BN/SsN, MR/N, BUF/N, M520/N, WN/N, ACI/N, WKY/N, and F344/N). To produce an HS a random breeding scheme was set up for about 60 generations creating a mosaic of founder variants allowing the fine-mapping of QTLs. In the HS rats used in paper IV the mapping resolution for a QTL was estimated to less than 3Mb¹⁰⁷ and could potentially in some genomic regions allow the identification of a single gene responsible for a phenotype.

4.2 Identifying disease causing genes in humans

Identification of genes regulating disease in humans was originally based on familial linkage studies where regions linked to disease usually range from 2-10Mb in span. Due to the low resolution the identification of genes using familial linkage studies has not been efficacious in complex diseases. Thus, genome- wide association studies (GWAS) where loci of 10- 100 kb can be identified has been favored instead¹⁰⁸. Here, one takes advantage of the fact that polymorphisms are not inherited independently, but together in linkage disequilibrium (LD) blocks. Around one million SNPs called tagged SNPs, representing different LD blocks across the genome is selected and genotyped in affected and non-affected individuals. Due to the number of markers used and number of individuals in GWAS studies, associations need to be corrected for multiple testing to exclude false positives. Thus, high p-values of $>10^{-8}$ is needed for genome-wide significans. Although many loci have been identified using GWAS, a large part of heritability called the missing heritability, cannot be accounted for¹⁰⁹. For example, the heritability in RA is estimated to be ~65% from studies in twins however in a recent GWAS study, the identified loci can only account for 50% of the heritability⁶⁶. This could be due to missing rare variants but also the fact that the genetic markers used, SNPs, might not pick up other types of important variants such as CNVs or insertions and deletions. Correct categorization of the phenotype studied is also important as the possibility of mixed types of disorders might mask a positive association. This is illustrated in paper II where the need for stratification based on serology is necessary to identify association to the *VAV1* gene.

5 PRESENT INVESTIGATIONS

5.1 Paper I

Spontaneous mutation reveals Endophilin A2 as a major regulator of autoreactive T cells and a potential new target in autoimmune disease

In this study we identified Endophilin A2 (EA2), a previously unknown and unique target, for treatment of autoimmune diseases. The importance of the gene in immune regulatory pathway was discovered as a consequence of a spontaneous mutation that occurred in our colony of rats rendering the normally highly susceptible DA rat resistant to induction of arthritis. The mutation inhibits transcription of the gene encoding EA2 making the rat a natural knockout. To cross-species confirm EA2's role in protection against arthritis we used gene knockout technology in mouse. The gene encoding EA2 is expressed in, and affects, many different leukocytes. However, the arthritis protection seen in EA2 deficient animals is mainly mediated via T cells. The T cell dependency was shown through pristane- primed CD4⁺ T cell transfer experiments and the reconstitution of T cell knockout mice with EA2 deficient and wild type thymocytes and subsequent arthritis induction. Investigating the EA2 effect on T cells in light of its molecular function, we saw that the EA2 deficient T cells are unable to internalize their T cell receptor to the same extent and thus are unable to proliferate at the same rate compared to normal wildtype T cells. The reduced T cell function leads to a decrease in autoreactive T cells and results in unsuccessful induction and subsequent blockage of disease progression. We could also show that the EA2 expression was increased in RA patients and thus propose a new interesting pathway in RA by which the activation of T cells can be modulated using inhibitors of EA2.

5.2 Paper II

VAV1 regulates experimental autoimmune arthritis and is associated with anti-CCP negative rheumatoid arthritis

The *Vav1* gene had previously been described to regulate severity in an animal model of multiple sclerosis and found to be associated in multiple sclerosis (MS)¹¹⁰. In this study we use different animal models for RA and genetic association studies to investigate the role of *Vav1* in arthritis. We immunized DA.BN-R25 congenic rats harboring a coding variant in the *Vav1* gene and DA littermates for CIA and PIA. While no effect could be observed in CIA a significant reduction in arthritis severity was found in PIA. As discussed above B cell dependency in pathogenesis in PIA and CIA seem to differ. Studies on depletion of T cells before and after established disease in adjuvant arthritis and CIA further confirm this observation¹¹¹. While depletion of T cells during priming is beneficial in both adjuvant arthritis and CIA, depletion after established disease reduce arthritis severity only in adjuvant arthritis while no ameliorating effect is observed in CIA. This suggests that after the B cells have been primed by T cells they no longer need T cells in order to propagate the disease. These experiments together with the observed reduction in T cell proliferation seen in DA.BN-R25 rats¹¹⁰ explain why we only could observe regulation by *Vav1* in PIA and not CIA. In the RA case-control studies only a weak association in the total population could be observed. However, when stratifying the RA patients to ACPA positive and ACPA negative, a stronger association could be found for the ACPA negative while no association was found in ACPA positive patients. In both PIA and ACPA negative RA the disease progression is thought of as being less dependent on antibodies and in these diseases T cells could have a more prominent role. Thus taken together our results indicate that *Vav1* regulates a T cell dependent mechanism in arthritis. Our results further illustrate the common pathways shared by different autoimmune disorders and the heterogeneous population of RA patients.

5.3 Paper III

Positioning of a polymorphic quantitative trait nucleotide in the *Ncf1* gene controlling oxidative burst response and arthritis severity in rats

The *Ncf1* gene regulates production of ROS by the phagocyte NADPH oxidase complex and has previously been associated with arthritis severity in DA.E3- *Ncf1* congenic rats. Interestingly the arthritis protective allele from the E3 rat increase the production of ROS which mediate protection. Three coding SNPs in the *Ncf1* gene differed between the susceptible DA rat and the resistant E3 rat. To understand the molecular mechanisms underlying the effect on arthritis, we needed to identify the arthritis causative SNP. Mutated recombinant *Ncf1* at the three different positions were tested for their effect on ROS production in vitro. The SNP responsible for an amino acid shift at position 153 from methionine to threonine was shown to restored the *Ncf1* mediated ROS production. To test the functional impact of this SNP on arthritis development, inbred rat strains were screened for polymorphisms in the *NCF1* gene. A sub-strain of the Wistar rat was identified with DA alleles at two of the three SNPs found to differ between DA and E3. Only the third SNP causing the amino acid shift at position 153 was identical to the E3 rat. The genomic region containing the Wistar allelic version of the *Ncf1* gene was isolated in a congenic strain and immunized for arthritis. Similar to the E3 rat, the new DA.Wistar-*Ncf1* congenic also showed reduced arthritis severity highlighting the 153 snp as the only disease regulating genetic variant. Thus we could functionally prove that the reduced arthritis severity observed in *Ncf1* congenic rats was due to increased ROS production and regulated by a single nucleotide.

5.4 Paper IV

Combined sequence-based and genetic mapping analysis of complex traits in outbred rats

Positionally cloning of genes is a time consuming and a costly endeavor. In the fourth study we utilized the NIH heterogeneous stock for high resolution mapping of 2000 rats outbred rats and collected 160 phenotypes to identify genes to complex traits involved in for example metabolism, immune regulation and cardiovascular disease. Eight founder strains of the HS rats were sequenced and their sequence imputed to haplotypes into 1400 SNP typed outbred rats used in the study. The strategy was proven successful and we could identify 35 causal genes involved in 31 phenotypes.

With the use of flow cytometry we studies the frequency of different leukocytes and expression of surface molecules in blood of naïve NIH-HS rats. Of interest to our work was the identification of the *Tbx21* gene in a QTL regulating the proportion of CD4⁺ cells with high expression of CD25. Here the candidate variant in the *Tbx21* gene leads to a glycine to arginine substitution at position 175 of the *Tbx21* and could possibly alter the DNA-binding domain of this protein. The *Tbx21* gene has been implicated in the genetic control of regulatory T cells previously and thus the QTL regulating CD4⁺CD25 high cells might represent regulatory cells. Further investigation and isolation of this variant is needed to conclude an association to the observed phenotype.

6 CONCLUDING REMARKS

Lack of efficient therapeutics in RA patients leads to high socioeconomic costs and severely reduce the quality of life for the individual¹¹². In addition, poor management of co-morbidities and an increased risk of infections with current therapies lead to an increased mortality in RA patients. Thus, there is a high unmet need in RA and further understanding of the disease pathogenesis is needed to discover new therapeutics.

Hypothesis free discovery of genes involved in autoimmunity using experimental crosses is a valuable tool to discover new therapies. The identification of the ROS regulating effect of the Ncf1 gene in autoimmunity has led to the development of ROS inducers for treating autoimmune disorders and have been shown to work in arthritis in rats¹¹³. We hope that the discovery of EA2 can lead to new and more effective therapies in autoimmune disorders too. By inhibiting EA2 there is a potential of addressing many of the unmet needs in RA. With the use of EA2 inhibitors we could potentially address two important features of RA, lymphocyte activation and target tissue destruction. First by inhibiting the activation of autoreactive T cells, which have been linked to the detrimental chronic circle of immune cell activation, stopping the autoreactive response. Second, through publically available data repositories (BioGPS) we have found that the gene expression of EA2 is increased in synoviocytes of RA patients compared to healthy controls and individuals suffering from osteoarthritis. EA2 has also been shown to regulate the endocytosis of membrane bound metalloproteinases¹¹⁴, known to be important for degradation of the extra cellular matrix. Thus by targeting EA2 in synoviocytes one can reduce their proliferation and inhibit their invasiveness leading to a subsequent regeneration of the target tissue. Additionally, RA patients have an increased risk of malignancies¹¹⁵ and it is still debated whether current treatments contributing to this¹¹⁶. Knocking down EA2 in cancer cells have shown to reduce proliferation and tumor invasion¹¹⁷ thus inhibiting EA2 in RA patients one could potentially also reduce the prevalence of malignancies.

In summary, the results of this thesis show that RA is indeed a heterogeneous disease and identification of genes could benefit from stratification, there is an overlap in disease pathways between different autoimmune diseases and that peripheral tolerance mechanism mediated by Endophilin A2, Vav1 and Ncf1 are crucial in limiting an autoimmune response.

7 FUTURE PERSPECTIVES

Positionally cloning of genes using animals is a powerful tool to functionally characterize and study genes involved autoimmune disorders. Although proven successful in this thesis, the identification of genes using F2 crosses and isolation in congenic strains is a time consuming endeavor. With the introduction of new technologies in the 'omics era and the drop in prices of sequencing, direct studies in humans comparing genotype to phenotype will probably replace many of the animal studies. However, the need for animal models will not decline, as many of the findings in humans will need functional characterization and validation in animals. Additionally, the need for many different animal models mimicking different subgroups, as evident in with the *VAV1* gene, will be valuable when developing new therapeutics.

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